THE EFFECTS OF OXYGEN SUPPLY WHEN IMMERSED ON THE THERMAL LIMITS AND PERFORMANCE OF THE WAVE ZONE ECHINODERM *COLOBOCENTROTUS ATRATUS*

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ABSTRACT

Thermal limits of marine ectotherms are hypothesized to be limited by oxygen demand. This limit arises from an animal's metabolic demand outpacing oxygen supply and delivery with increasing temperatures. We applied this theory to the intertidal zone, where animals are exposed to extreme temperature ranges and emersion, to examine how oxygen delivery capacity can limit vertical distributions of organisms. We tested the upper thermal limit of performance (survival and attachment strength) in a wave-zone obligate urchin, *Colobocentrotus atratus,* under both ambient (21%) and enriched (35%) O_2 conditions, to determine if these urchins are limited by oxygen availability in water when exposed to temperatures ranging from 25 to 37ºC. Survival when thermally stressed was increased by enriched O_2 conditions, indicating oxygen limitation of *C. atratus* under submerged conditions. Attachment strength declined with increasing temperature, but was not affected by O_2 enrichment. Our data support that the lower limit on vertical distributions of *C. atratus* are set by abiotic conditions resulting from oxygen limitation when submerged.

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INTRODUCTION

Rocky intertidal organisms experience extreme environmental variability in temperature and oxygen availability throughout tidal cycles. At low tides, temperatures can reach physiologically stressful levels (Cox and Smith, 2011) and during high tide or submersion, the amount of oxygen that is available for use by organisms is significantly reduced (Truchot, 1990). Temperatures outside of an organism's functional range can have detrimental impacts on performance (Bjelde and Todgham, 2013; Dahlhoff and Somero, 1993; Dowd et al., 2015; Stillman and Somero, 1996), species interactions (Sanford, 1999; Wethey, 1984), reproduction (Willett, 2010), and survival (Denny et al., 2006; Miller et al., 2009; Schneider et al., 2010; Stillman and Somero, 2000; Tomanek and Somero, 1999). Thermal stress has long been considered the dominant stressor in the intertidal (Somero, 2002); however, a major component of thermally-limited performance is driven by the effects of temperature on the dynamics of oxygen supply and demand (Pörtner, 2001). For ectotherms, temperature increases cause metabolic oxygen demand to rise (Pörtner, 2001), relying on delivery rate and the availability of oxygen to meet this demand (Verberk et al., 2016). Pörtner et al. (2001) put these two concepts together in the oxygen capacity limited thermal tolerance (OCLTT) hypothesis, which posits that at temperatures outside an optimal range, limitations of organismal performance are caused by oxygen supply not meeting metabolic demand under thermal stress. Studies on aquatic organisms have provided the strongest evidence supporting the OCLTT hypothesis (Verberk et al., 2016). Aquatic organisms face limited aerobic respiration potential compared to their terrestrial counterparts, due to reduced availability of oxygen in water (Bjelde et al., 2015; Verberk et al., 2011; Verberk et al., 2016). In water there is a 30 times lower concentration of oxygen and 200,000 times (at 20°C) decrease in diffusivity of oxygen resulting in increased

ventilation effort (Giomi et al., 2014; Truchot, 1990; Verberk and Atkinson, 2013). This increase in effort coupled with a decrease in efficiency of ventilatory mechanisms while submerged, raises an organisms baseline metabolic rate and limits overall aerobic scope for organismal performance (Giomi et al., 2014). When temperature increases, and metabolic demand for oxygen rises, submerged organisms are limited by their ability to acquire enough oxygen to deal with thermal stress (Giomi et al., 2014; Mark et al., 2002; Pörtner et al., 2006; Verberk et al., 2011). Submersion under thermal stress presents an oxygen limited scenario for intertidal organisms that are regularly air exposed (Bjelde and Todgham, 2013; Bjelde et al., 2015; Fusi et al., 2016).

One method of testing the OCLTT hypothesis is to experimentally raise oxygen levels and determine whether increased O_2 can "rescue" performance of organisms under thermally stressful conditions (Pörtner et al., 2006). Similar rescue experiments have been performed in a range of taxa including Antarctic bivalves (Peck et al., 2007; Pörtner et al., 2006), temperate gastropods (Bjelde et al., 2015; Gardeström et al., 2007; Vosloo et al., 2013), crustaceans (Bowler, 1963), and fish (Bagherzadeh Lakani et al., 2013; Berschick et al., 1987; Foss et al., 2003; Mark et al., 2002). The temperate intertidal limpet *Lottia digitalis* demonstrates that oxygen enrichment increases maximum heart rates but not cardiac breakpoint temperatures or flat-line temperatures (Bjelde et al. 2015). The rescue of one aspect of cardiac performance, maximum heart rate, in *L. digitalis* provides evidence of oxygen limitation at ambient oxygen concentrations while submerged. Determining if other intertidal organisms are oxygen limited when submerged could also provide key insight into how physical or physiological pressures set their vertical distributions (Stillman and Somero, 1996). Two species of temperate intertidal *Petrolisthes* crabs inhabit distinct vertical zones, high and low, due to physiological,

biochemical, and morphological adaptations to aerial respiration and submerged respiration respectively (Jensen and Armstrong, 1991; Stillman and Somero, 1996).

In the Hawaiian archipelago, the rocky intertidal is not characterized by the large tidal changes observed in temperate regions; rather, the Hawaiian intertidal has three zones - the emergent (upper), wave (middle), and submergent (lower) zones (Bird et al. 2013). The emergent intertidal zone experiences tidally-influenced wave action, air exposure, and wide temperature fluctuations; organisms in the submergent intertidal, in contrast, are fully submerged at high tide, washed by waves at low tide, and experience negligible thermal stress. The middle "wave zone" experiences constant wave action across all tidal cycles (Bird et al., 2013). While there have been studies on wave swept habitats in Hawai‘i focusing on how hydrodynamic stress from wave action impacts organisms and their interactions (Denny, 1995; Denny and Gaylord, 1996; Gallien, 1987; Santos and Flammang, 2008), the impact of other physical and physiological stressors, such as temperature and oxygen, have received little attention (Bird, 2006; Cox and Smith, 2011). Organisms in the exposed Hawaiian intertidal experience a mosaic of temperatures with a 20°C range across the habitat (Cox and Smith, 2011) and substratum temperatures can increase from 15°C to 40.5°C over a period of 12 to 24 hours (Bird, 2006). The traditional paradigm of intertidal zonation is that abiotic or physical and physiological factors set upper vertical limits and biotic or ecological factors set lower vertical limits (Connell, 1961). These distinctions may be an oversimplification of the complexity of abiotic and biotic stressors that influence organisms in the intertidal (Tomanek and Helmuth, 2002), because an interplay of environmental factors and ecological interactions are known to drive the distributions of various intertidal organisms (Sanford, 1999; Wethey, 1984). Some lower limits have been observed to be set by abiotic factors including substrate composition in intertidal crabs

of the genus *Petrolithes* (Jensen and Armstrong, 1991) and by wetness in intertidal barnacles of the genus *Chthamalus* (Power et al., 2001). However, these occurrences of lower vertical limits set by abiotic factors are unusual. This may be especially true in wave zones, like Hawai‘i, where biotic interactions, which are typically thought to set lower distributional limits of intertidal organisms (Connell, 1961; Paine, 1974), are reduced due to constant hydrodynamic stress that reduces the effectiveness of predation and competition (Bird et al., 2013). The wave-dominated zone of Hawai‘i provides an opportunity to test how physiological stressors, such as temperature and oxygen, could serve as abiotic constraints on the lower vertical limits of intertidal organisms.

Colobocentrotus atratus Linnaeus, 1758 is an urchin that exclusively inhabits the wave-swept rocky intertidal zone. These urchins have a distributional range that includes the Hawaiian island chain and parts of the tropical Indo-Pacific (Mortensen, 1943). *C. atratus* or "Hā‘uke‘uke" is a valued traditional food source of native Hawaiian people (Newman, 1970) and an ecologically important species. The grazing pressure of *C. atratus* helps shape the community structure in the Hawaiian rocky intertidal; the intertidal mollusc *Cellana sandwichensis*, or opihi, relies on an indirect-commensal relationship with *C. atratus* because grazing of turf and macroalgae by the urchin, makes grazing space available for the opihi (Bird, 2006).

Typical sea urchin morphology, consisting of radially projecting tube feet and spines, is poorly designed for aerial respiration and high wave action (Burnett et al., 2002; Denny and Gaylord, 1996). However, *C. atratus* possess a unique morphology (Fig. 1), are found exclusively in the wave zone, are exposed to air most of the time, and are never observed below the water line (Bird, 2006). *C. atratus*’s morphology is well adapted to physical stress from waves compared to many sympatric echinoids (Denny and Gaylord, 1996; Santos and

Flammang, 2008). The drag coefficient of *C. atratus* is similar to that of other urchins, but the oral-aboral flattening of its body and it's flattened pedestal-like spines reduce lift and lower acceleration forces these urchins experience (Denny and Gaylord, 1996). Similarly, *C. atratus* have greater tube foot attachment strength and use more tube feet when attaching to surfaces than more typical subtidal urchins (Santos and Flammang, 2008). The unique morphology of *C. atratus* may also play a role in enhancing desiccation resistance, thermal tolerance, and oxygen uptake while emersed (Denny and Gaylord, 1996; Gallien, 1987; Santos and Flammang, 2008). Aerial exposure poses a challenge for gas exchange in typical sea urchins because their primary respiratory surface, the tube feet, collapse in air leading to a substantial reduction in function (1/5 to 1/7 of submerged levels) (Fenner, 1973; Johansen and Vadas, 1967). The pedestal-like aboral spines of *C. atratus* appear to provide desiccation resistance (Fig. 1), while most of the tube feet are located on the oral surface where a moist environment can be maintained for gas exchange with the air (Santos and Flammang, 2008). These unique morphological adaptations have advantages when emersed, but when submerged this same morphology may restrict respiratory tube feet, limiting the animal's ability to exchange gases in an already reduced oxygen environment.

To understand the role of available oxygen in limiting the vertical distribution of *C. atratus*, we examined performance and survival at different combinations of oxygen and temperature under submerged conditions in the laboratory. Urchins were exposed to five temperature treatments to determine if thermal stress will negatively affect the submerged performance of *C. atratus*. Temperatures were selected to represent a range from average ocean temperature (25°C) up to the maximum recorded substratum temperature (38°C) of the Hawaiian intertidal (Cox and Smith, 2011). To test whether oxygen can "rescue" performance at high

temperatures, we tested for effects of temperature under two oxygen levels, 21% and 35% air saturation.

METHODS

Urchin Collection

Adult *C. atratus,* mean longest test diameter 20 to 53 mm, were collected at low tides from rocky shoreline at Point Panic, O‘ahu (21°17'26.29"N, 157°51'41.50"W) on five dates in 2016: August 4th, September 29th, October 14th, November 4th, and December 2nd. Individual urchins were selected haphazardly and dislodged by hand from rock substrate using a twisting motion to minimize damage to their tube feet, test, and spines. Urchins from each of the five collecting dates were used in one of five replicate experiments described below. After collection, urchins were kept in flow-through seawater at Kewalo Marine Laboratory in Honolulu, HI under common-garden conditions for a minimum of 7 days, but no more than 10 days before experiments. The common garden consisted of open-air flow-through sea tables in which we modified the inlet hose to provide a constant seawater spray inside the sea-table. This was done by drilling holes around all sides of the last 3 inches of the inlet hose and capping the end. Prior to the introduction of urchins, algae were allowed to grow on all surfaces of the sea tables for a week to provide a source of food. The sea-tables were outside, so urchins received a natural light/dark regime with daily temperature fluctuations. The daily temperature of the incoming seawater in the sea-tables ranged from 25.3 to 27.7°C (National Oceanographic and Atmospheric Administration’s National Ocean Service, ID# 1612340, http://www.ndbc.noaa.gov/). Experiments were performed on the University of Hawai‘i at Mānoa campus.

Temperature and Oxygen Treatments

To determine if increased oxygen availability and temperature affected the performance of *C. atratus*, attachment strength of urchins was recorded after exposure to different combinations of temperature and oxygen saturation treatments. The experimental treatments exposed urchins to five temperatures (25°C, 28°C, 31°C, 34°C, 37°C) and two oxygen concentrations (ambient, 21%; enriched, 35% for a total of 10 treatments (Fig. 2). Over the course of the experiment, five sequential replicate runs were performed with 120 urchins each; in each run, 12 urchins were exposed to each of the 10 oxygen x temperature combinations (such that each oxygen x temperature treatment was replicated five times) (Fig. 2). No urchin was used for more than one treatment or run, thus a total of 600 total urchins were used in the combined study.

Experiments were performed in two large standing incubators (VWR #10753-894, VWR International): one incubator served as the ambient oxygen control and the other as the oxygen enriched treatment. The two experimental atmospheric oxygen concentrations were 21% (ambient) and 35% (O_2-enriched). The gas concentration in the "ambient" incubator was not manipulated. To achieve the O_2 enriched environment, we used a ROXY-1 O_2 & universal controller (Sable Systems International, Las Vegas, Nevada) attached to a compressed gas cylinder of 100% oxygen to raise the concentration of O_2 in the air inside one incubator to 35% (Fig. 3). Each incubator had a pair of aquarium pumps that bubbled the gas inside the incubator into individual treatment jars for the duration of the experiment (Fig. 3).

The five experimental exposure temperatures were 25°C, 28°C, 31°C, 34°C, and 37°Che order in which temperature treatments were applied was randomly chosen before the start of each of the five runs. One target temperature was tested per day (at two O_2 concentrations) such that the ten experimental O2/temperature combinations were all completed over a five-day period.

Starting at our control temperature of 25°C, the temperature of each incubator was increased at a rate of 5±0.5°C per hour until it was within 3°C of the target temperature, at which point the incubator slowed until the exact target temperature was reached. The rate of temperature increase was chosen to reflect natural intertidal heating rates observed in the Hawaiian intertidal (Bird, 2006).

Prior to experiments, each individual urchin was measured for test height and two diameters, the longest and its perpendicular. Each urchin was then placed in an individual 500 mL specimen jar filled to the top with 1µm filtered seawater. Each jar had two small openings in the lid, one to allow for the aeration tube and the second to relieve any pressure that may build up from aeration. Urchins were allowed to acclimate at 25°C in aerated jars for 30 minutes prior to beginning the experiment (all urchins attached to the side of their jars during this period). This treatment occasionally induced spawning, and any urchins that visibly released gametes in their chamber over the course of the experiment were excluded from analyses (four urchins in run #4 and two urchins in run #5).

The flow of O_2 gas was started prior to the beginning of each experiment, immediately after animals were introduced. After the acclimation period, the incubator was set to ramp up to the target temperature. Incubators were set to decrease the temperature down to 25°C by end of the exposure period. This was carried out using the incubator's cooling feature that reduced the temperature at a rate of 4.5±0.5°C per hour. Urchins were exposed to treatments for a total of 24 hours, which included ramp-up and ramp-down. Estimated ramp times were: 28°C, 36min; 31°C, 1h 12 min; 34°C, 1h 48min; 37°C, 2h 24min. Estimated cooling times were: 28°C, 40min; 31°C, 1h 20min; 34°C, 2h; 37°C, 2h 40min. Because of different ramp and cooling times,

temperature exposure period was estimated to vary by temperature: 28°C, 22h 44min; 31°C, 21h 28 min; 34°C, 20h 12min; 37°C, 18h 56min.

Measurements of realized temperature and oxygen treatments in experimental chambers

Because both temperature and oxygen were manipulated in the air in the incubator, rather than the water containing the urchins, we ran trials to measure the water temperature and air saturation under experimental conditions. To measure temperature, temperature dataloggers (HOBO TidbiT v2, Onset Computer Corporation, Bourne, Massachusetts) were set to record every five minutes and placed in three randomly selected chambers with urchins at the start of each treatment. Temperature traces were used to assess effective ramping rates and temperature stability in the jars.

To determine whether oxygen concentration in the experimental jars matched our target levels, dissolved oxygen concentration of the seawater in nine randomly selected test chambers was measured after jars containing animals had completed the exposure period for their experimental target temperature (just prior to the cool down period). Dissolved oxygen concentration was measured with a handheld oxygen electrode probe (YSI 550A, YSI Inc., Yellow Springs, Ohio). Both dissolved oxygen concentration (in mg/L) and percent air saturation values were recorded, correcting for local atmospheric pressure, salinity, and incubator temperature of each recording. The oxygen probe was calibrated using an atmospheric pressure set at 1 atm (UHMET, weather.hawaii.edu) and a salinity of 35 ppt, determined using a portable refractometer with a 0-100% salinity range.

Survivorship and Lethal Temperature

Mortality of *C. atratus* was measured after allowing urchins to recover at 25°C for 1 h post attachment strength measurements. Urchins were scored as dead when they failed to reattach to the side or bottom of the jar by the end of 1 h (described below). LT_{50} (temperature at which 50% of the urchins failed to reattach) was calculated for both oxygen treatments in each of the five runs by fitting the mortality data to a logarithmic regression (Litchfield, J. T. and Wilcoxon, 1949) in Sigmaplot 12.5 statistical software (Systat Software Inc. 2011) and determining LT_{50} using Sigmaplot's reverse prediction function.

Attachment Strength

After the 1 h recovery period and before scoring survivorship, the attachment strength of each urchin was measured as an estimate of performance. Attachment strength was measured using the force required to remove an urchin from a glass surface. Measurements of attachment strength were performed using application of lateral pressure (Santos and Flammang 2007). Using an analog force gauge (Shimpo Model MF-20, Electromatic Equipment Co, Cedarhurst, New York) with 88N range and 0.1N sensitivity, steady constant pressure was manually applied to the side of each individual urchin as it was attached to the wall of the jar. Constant downward pressure was applied until the urchin was fully dislodged from the wall of the jar, which typically occurred as a sudden attachment failure. The force gauge was set to record peak force in N, which occurred immediately prior to detachment. All measurements were performed by the same operator to ensure consistency. Because numbers of tube feet, which directly determines attachment strength, has been shown to be related to urchin size in *C. atratus* (Santos and Flammang, 2008), we also measured urchin volume. Volume of each urchin was determined

using the long (a) & short diameter (b), and test height (c) measurements. Volume of the urchin (V) was modeled as half an ellipsoid resembling a dried *C. atratus* test:

$$V = \frac{(4/3)\pi abc}{2}$$

Urchins that were no longer able to attach after the end of exposure were scored as dead and excluded from the attachment strength analysis.

Statistical Analyses

Following the approach of Dingemanse & Dochtermann (2013), a linear mixed effects model was used to test the effects of oxygen and temperature on survival of *C. atratus*. Fixed effects were oxygen, temperature, and their interaction. Run (#1-5) was included as a random effect. All proportion data were arcsine transformed before analysis to reduce variance and normalize the data. To determine whether there were significant differences among temperature and oxygen treatments we used a Tukey HSD post hoc test.

LT_{50} estimates were paired by oxygen treatment for each of the five runs. Differences in LT_{50} estimates between oxygen treatments were compared using a paired t-test (n=5 per oxygen treatment; ambient and O_2-enriched).

A separate linear mixed effects model was used to analyze attachment strength across treatment groups. Fixed effects included oxygen, temperature, and run (#1-5), with urchin volume included as a fixed effect and covariate. The two-way interaction between oxygen and temperature was also analyzed, but we were unable to analyze further interactions due to limited power. Preliminary analyses found there were differences in mean urchin volume between runs and oxygen treatments, and a significant positive relationship between attachment strength and urchin size. Therefore, urchin volume was included as a covariate to account for variation in the

size of individuals tested. The model included run (#1-5) nested within temperature x oxygen concentration (10 combinations) as a random effect. This random effect allows us to account for the structure of our experiment in the model, with individual urchins as subsamples in each incubator in each run (Bolker et al., 2009). Attachment strength and urchin volume measurements were log transformed to meet the assumptions of normality of the residuals. Differences between the control temperature of 25°C and each temperature increase were examined for significance using a Dunnett-Hsu post-hoc test. Differences between runs (#1-5) were compared using a Tukey HSD post-hoc test. All statistical analyses were run in JMP Pro statistical software package (v13 SAS Institute Inc.).

RESULTS

Measurements of realized temperature and oxygen treatments in experimental chambers

The large standing incubators reached target temperature within 3.67 - 4.5 hours of the start of ramping and took 45 min to 2.5 hours to reduce the temperature back to 25°C by the end of the experimental period (Fig. 4). When each incubator approached within 3°C of the target temperature the rate of increase slowed (Fig. 4). The ramping rate for 28°C treatment and the last 3°C increase for 31°C, 34°C, and 37°C treatments was 0.75 to 1.5°C per hour. In 31°C, 34°C, and 37°C treatments the ramping rate was within 0.5°C of our target rate of 5°C per hour prior to the final 3°C increase. We only achieved the target rate of 5°C in the higher temperature treatments due to a limitation in the incubators that would reduce the rate of heating when within 3°C of the target temperature. The lower recorded ramping rates are still within natural temperature increases observed in the Hawaiian intertidal (1°C h-1; Bird, 2006) and do not extend the time it takes to achieve treatment temperature, reducing the effect of confounding

factors from extend temperature ramps (Terblanche et al., 2011). The time each treatment took to reach the target temperatures varied: 28°C, 3h 45min; 31°C, 4h 15min; 34°C, 4h 15min; and 37°C, 4h 30min. The cool down rate for each temperature treatment met our target of 4.5°C (Fig. 4). The time each treatment took to reach 25°C varied: 28°C, 45min; 31°C, 1h 20min; 34°C, 2h; and 37°C, 2h 35min. Measured temperatures were all within ±0.5°C of expected values. Animals in different temperature treatments were thus exposed to target temperature for between 16.5 and 19 hours (Fig. 4).

Oxygen concentration remained at 100% saturation and at target levels in both oxygen treatments across all temperatures (Fig. 5A). Oxygen concentration in mg/L declined (as expected, Benson and Krause 1984)) with increasing temperature in both oxygen treatments (Fig. 5B).

Survivorship and Lethal Temperature

Both temperature and oxygen had statistically significant effects on survival of *C. atratus* and there was a statistically significant interaction between temperature and oxygen (Table 1, Fig. 6). Survival differences between oxygen treatments at 34°C and 37°C were statistically significant (Tukey HSD, p=<0.0001 at both temperatures), but this effect was not statistically significant at the lower temperatures. Oxygen enrichment increased survival by 23% compared to ambient, and this effect was statistically significant (Table 1). At 25°C and 28°C urchin survivorship was >90% in both treatments (Fig. 6). In ambient O_2, survivorship was lower at 34°C than O_2-enriched treatments (Tukey HSD, p=0.0002), which had >90% survivorship up to 34°C (Fig. 6).

The effect of oxygen treatment on LT_{50} was marginally statistically significant in a paired t-test (t-ratio=2.78, p=0.05). LT_{50} increased from 33.9°C under ambient conditions to 36.3°C under the O_2-enriched treatment (Fig 7), for a mean increase in LT_{50} of 2.4°C.

Attachment Strength

Urchins had lower attachment strength at higher temperatures across both oxygen treatments, and this effect was statistically significant (Table 2, Fig. 8). Urchins exposed to 34°C and 37°C showed significantly lower attachment strength (Fig. 8, Dunnett-Hsu, p=<0.001, p=0.003). Attachment strength in the O_2-enriched treatment was approximately 15-22% higher when compared to ambient O_2 across all temperatures except the 37°C treatment (Fig. 8). In the ambient oxygen treatment, all but one urchin died at 37°C in all five trails. There was no statistically significant effect of oxygen or the interaction between oxygen and temperature on urchin attachment strength (Table 2).

Run and volume both had statistically significant effects on attachment strength (Table 2). There were statistically significant differences between runs in attachment strength: the attachment strength of urchins from run #5 was significantly higher than in runs #1 and 2 (Fig. 9, Tukey HSD, p=0.0165, p=0.0048, respectively). There was a statistically significant positive relationship between attachment strength and urchin volume for temperatures 25°C to 34°C in both oxygen treatments (Fig. 10). In the 37°C in O_2-enriched conditions there was no clear increase in attachment strength with size (Fig. 10). As above, we were unable to assess the interactive effects of size and oxygen concentration at 37°C because of high mortality under ambient O_2 at that temperature. At 34°C in ambient O_2 and 37°C in O_2-enriched conditions there was no trend of attachment strength increase with urchin volume (Fig. 10).

DISCUSSION

Survivorship and Lethal Temperature

We found that survival, one of our indices of performance, was rescued by oxygen supplementation when urchins were submerged and thermally stressed, but attachment strength was not. This suggests that at higher temperatures, animals in some of our experimental treatments experienced a mismatch between oxygen supply and demand that affected survival, an outcome predicted by the OCLTT hypothesis (Pörtner, 2001; Pörtner, 2002). However, because attachment strength also decreased with increasing temperature was not rescued by supplemental O_2, this aspect of organismal performance is likely not constrained by the same oxygen-temperature dynamics as survival.

We found evidence for oxygen limitation of survival in submerged *C. atratus* at 34°C and an estimated thermal limit of 33.9°C in ambient O_2 and 36.3°C in enriched O_2. Although intertidal temperatures in air have been found to reach as high as 38°C in the habitat of *C. atratus* in Hawaii (Cox and Smith, 2011), water temperatures only reach a maximum of 32.6°C in this same area during the collection period (NOAA, ID# 1612340, http://www.ndbc.noaa.gov/). While this water temperature may be stressful to the urchins, it is cooler than the lethal limit in our 24-h exposures. Urchins are unlikely to experience such long periods of submersion in their natural habitat, and thus, water temperatures alone may not set the lower vertical limit of *C. atratus.* However, the increase in metabolic demand for oxygen at suboptimal temperatures may exceed the supply of oxygen than is available for *C. atratus* when submerged. A stressful effect of suboptimal temperatures is suggested by a decline in survival under normoxic conditions beginning at 31°C (Fig. 6). This decline indicates that while urchins may not reach their thermal

limits when submerged, they are negatively affected by oxygen-temperature interactions if continuously submerged at suboptimal temperatures during warmer months in Hawaiian waters.

Upper thermal limits can also vary between submerged and emersed conditions (Bjelde and Todgham, 2013). We suggest that the combination of greater oxygen availability and the potential capacity to use this oxygen have allowed *C. atratus* to inhabit the thermally and hydrodynamically stressful environment of the wave swept intertidal, but potentially at a cost of reduced performance in water. While we did not measure thermal limits in air, *C. atratus* might have higher thermal tolerance in air than water because oxygen diffusion rates in water are 300,000 times lower than in air (Verberk et al., 2011), water is 800x denser than air (Dejours, 1981) and has 30x less available oxygen (Truchot, 1990). Oxygen is therefore more limiting in water than air and organisms expend more energy when performing oxygen gas exchange in water (Verberk and Atkinson, 2013).

The capacity to use available oxygen is dependent on the mechanisms by which an organism exchanges respiratory gases. In general, tissue surface area (i.e. tube feet, oral gills) is critical to gas exchange in sea urchins (Fenner, 1973; Johansen and Vadas, 1967). In *C. atratus*, the efficiency of gas exchange could be compromised by the "shield" of flattened pedestal-like spines that cover most of the aboral surface. While no studies have been performed on the spine behavior of *C. atratus*, we observed that they raise and spread their pedestal-like spines during submersion which may allow oxygenated seawater to flow over epidermal tissue and aboral tube feet. This behavior may allow them to more effectively exchange gases while submerged, but may be costly due to increased drag. The pedestal-like spines may also aid in respiratory gas exchange by enhancing retention of extracorporeal water on the aboral surface of the test, allowing tube feet to continue to exchange gases while the animal is emersed (Denny and

Gaylord, 1996; Gallien, 1987; Santos and Flammang, 2008). An alternative form of aerial gas exchange seen in the sea urchin *Strongylocentrotus purpuratus* relies on a facultative lung formed inside their digestive tract with air (Burnett et al., 2002). When emersed and exposed to air, *S. purpuratus* releases fluid from their esophagus, termed emersion fluid, and their gut takes in air to fill the space opened by fluid loss (Burnett et al., 2002). The digestive tract of urchins is limited to performing respiratory functions when the animal is not feeding (Stott, 1955), as a result this is viewed as only a temporary method of aerobic respiration because *S. purpuratus* is not active during emersion (Burnett et al., 2002). Because *C. atratus* are almost always found emersed (though wave-washed), the gut seems unlikely to serve a respiratory function if such a function is incompatible with digestion of food. Likewise, constant exchange of gas between the gut and the air could increase desiccation, a stress to which most urchins are particularly sensitive (Burnett et al., 2002).

Attachment Strength

Unlike survival, we did not find a significant interaction between oxygen and temperature when attachment strength was the performance metric. According to the OCLTT hypothesis, overall organismal performance is expected to decline as thermal limits are reached because of limited aerobic scope from oxygen limitation (Pörtner, 2001). While our results did show a decrease in attachment strength with increasing temperature, this decrease could not be rescued by O2 supplementation.

These results contrast with previous studies of oxygen limitation on other intertidal organisms, including the limpet *Lottia digitalis* (Bjelde et al., 2015) and the Antarctic clam *Laternula elliptica* (Peck et al., 2007). *L. digitalis* did not show an increase in critical cardiac

thermal limits, but saw enhanced maximum cardiac performance under greater oxygen availability (Bjelde et al., 2015). Comparatively, the temperature at which *L. elliptica* were successful in burying themselves was increased by 0.7°C to 1.4°C when the oxygen content of water was raised (Peck et al., 2007). Both cardiac performance of *L. digitalis* and burying performance of *L. elliptica* focus are whole-animal functions that integrate across several levels of organization; in contrast, echinoderm attachment integrates the individual performance of tens to hundreds of tube feet that each obtain O_2 through diffusion and thus function as semi-independent respiratory structures (Fenner, 1973). If so, the attachment strength of individual tube feet may not be oxygen limited because the radius of an individual tube foot is small enough that it does not become diffusion-limited at high temperatures. Attachment in echinoderms is based on a combination of temporary surface adhesive and tube foot stem strength (Santos et al., 2009). It is this temporary surface adhesive and its de-adhesive counterpart that allows the animal to detach and move, and these processes may be negatively impacted by high temperatures (Ehrlich, 2010; Santos et al., 2009). The temperature sensitivity of tube foot adhesive secretions in *C. atratus* will need to be investigated to provide insight into how thermal stress affects attachment strength of the urchin as a whole.

Urchin size also significantly affected attachment strength across temperature and oxygen treatments. This trend was visible across all temperatures except for the 34°C and 37°C treatments in the ambient and enriched O_2 conditions respectively (at 37°C sample size was limited by high mortality in the ambient oxygen treatment) (Fig. 10). O_2-enriched conditions appear to allow larger urchins to perform better at 34°C than smaller urchins. We speculate this pattern suggests a thermal limit to attachment strength of *C. atratus* and that O_2 enriched conditions increase this limit. In prior studies on the attachment strength of *C. atratus,* larger

urchins had a greater number of tube feet, which in turn increased their potential attachment strength (Santos and Flammang, 2008). With increasing temperatures, it is important to consider that as the ratio of surface-area-to-volume decreases with size, all else being equal, larger animals will experience greater oxygen limitation (Atkinson et al., 2006). Peck et al. (2007) observed a similar effect of oxygen and body size on burying performance in the Antarctic clam *L. elliptica* and found that larger individuals had reduced burying performance at higher temperatures, and additional oxygen increased the temperature at which larger individuals could bury themselves normally.

In addition to oxygen and temperature effects, we found that animals from some runs were had significantly higher overall attachment strength than others (Fig. 6). One possible explanation is that animals for the different runs were collected at different times of year. Run #5, for which animals were collected in December, showed stronger attachment strength than other blocks for which animals were collected between August and November. One possible explanation is field acclimatization; animals that experienced warmer thermal regimes prior to collection could show improved lab performance under thermal stress (Stillman and Somero, 2015). Similar acclimatization-based patterns have been found in two other tropical sea urchins, *Echinometra lucunter* and *Diadema antillarum*, both of which showed increased righting performance at high temperatures when animals were collected during warm summer months compared to winter and spring (Sherman, 2015). However, *C. atratus* in run #5, which were the most thermally-tolerant group, were collected when ocean temperatures were comparatively cool, averaging 25.3°C (NOAA, ID# 1612340, http://www.ndbc.noaa.gov/). While we do not know the basis for the differences between runs, one possible explanation could be that high field

temperatures prior to collection of animals for the first four runs resulted in stressed animals with reduced lab performance.

Conclusions

While many intertidal organisms have vertical distributions limited on the upper end by temperature (Connell, 1961; Foster, 1971; Paine, 1974; Stillman and Somero, 2000; Wethey, 1984), our results show that the wave-zone urchin *C. atratus* are also be limited by temperature and oxygen at the lower end of their distributions. Thus, one potential factor setting lower limits for *C. atratus* is the oxygen limitation they experience while submerged. Field studies may shed light on how additional factors limit the vertical distribution of *C. atratus*: physical stressors, such as wave height, and biotic factors, such as food availability, competition, or predation. Vertical zonation patterns of intertidal organisms are likely to shift due to changes in physiological stress gradients and biotic interactions from anthropogenically driven climate change (Lubchenco et al., 1993). In addition to shifting zonation patterns, as water temperatures warm, *C. atratus* will become increasingly oxygen limited when submerged, reducing the length of time that animals can tolerate submersion. This effect will occur in concert with increased air temperatures, which will increase stress on animals at their upper vertical limit. The combined effects of warming water and air temperatures are likely to narrow the vertical distribution of *C. atratus*, thus reducing available optimal habitat and potentially impacting population sizes and geographic distribution; in addition, warming temperatures will reduce their attachment strength, which is a crucial fitness attribute in species that inhabit wave-swept tropical shores. Abiotic lower distributional limits are rarely considered in intertidal systems (Somero, 2002), but because adaptation to the aerial environment may increase the vulnerability of intertidal

organisms to submersion as global temperatures increase, these should be a key component of future studies into the effects of anthropogenically driven climate change on intertidal populations.

Table 1. Results of a linear mixed effects model on the effect of increased oxygen availability and increased temperature on survivorship of *C. atratus*

	Num *df*	Den *df*	F ratio	*P*
Temperature	4	36	77.29	**<0.0001**
Oxygen	1	36	30.80	**<0.0001**
Oxygen x Temperature	4	36	4.67	**0.0039**

Table 2. Results of a linear mixed effects model on the effect of increased oxygen availability and increased temperature on attachment strength of *C. atratus*

	Num *df*	Den *df*	F ratio	*P*
Temperature	4	41.3	9.79	**<0.0001**
Oxygen	1	198.3	1.11	0.2928
Oxygen x Temperature	4	41.4	0.21	0.9300
Run	4	30.3	4.50	**0.0057**
Volume	1	447.6	106.60	**<0.0001**

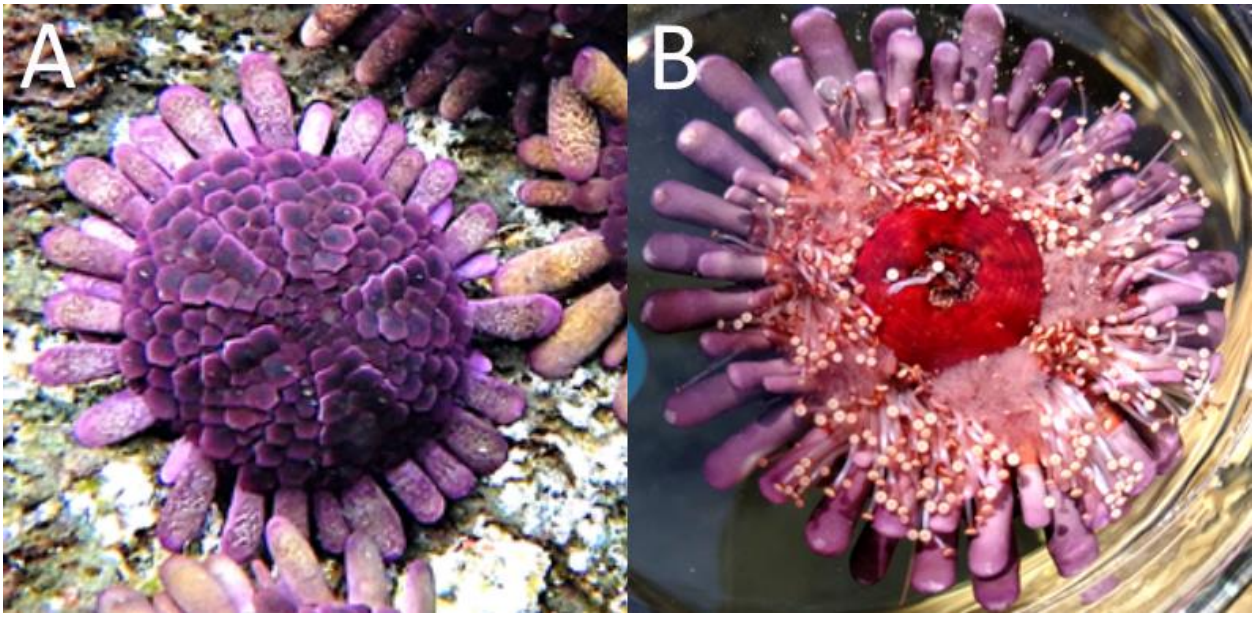

Figure 1. Photo of *C. atratus* morphology. **A.** Aboral view of pedestal-like spines and paddle-like spines. **B.** Oral view of paddle-like spines, tube feet, and mouth.

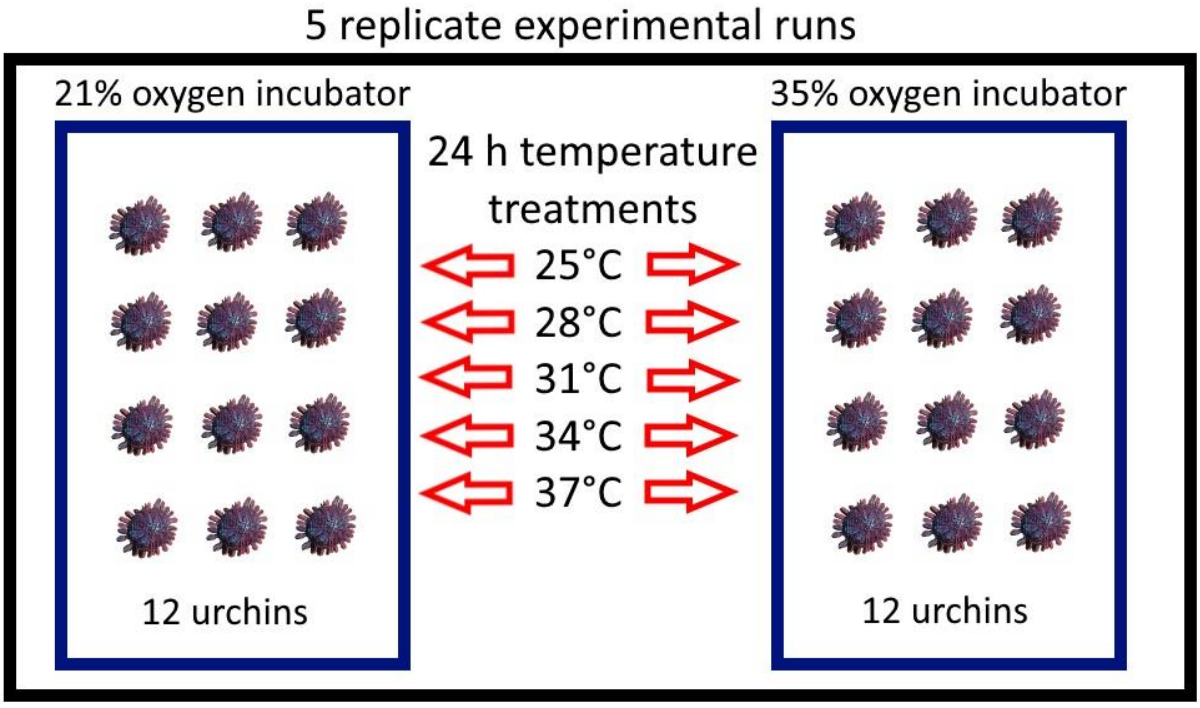

Figure 2. Schematic of experimental design.

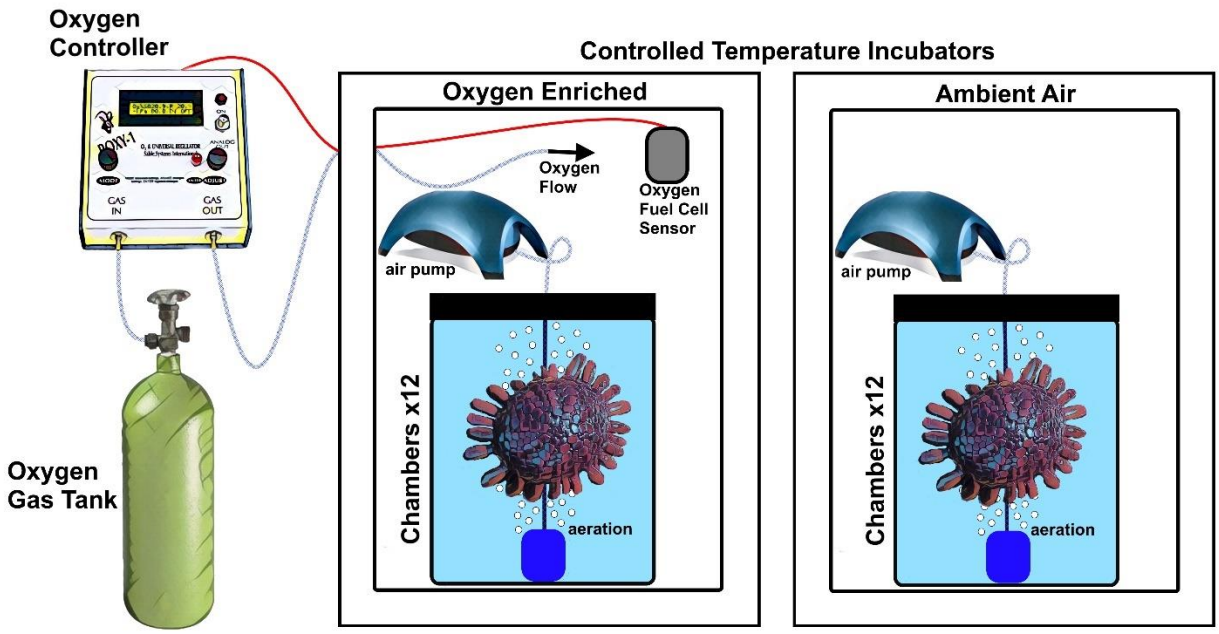

Figure 3. Schematic of incubator setup.

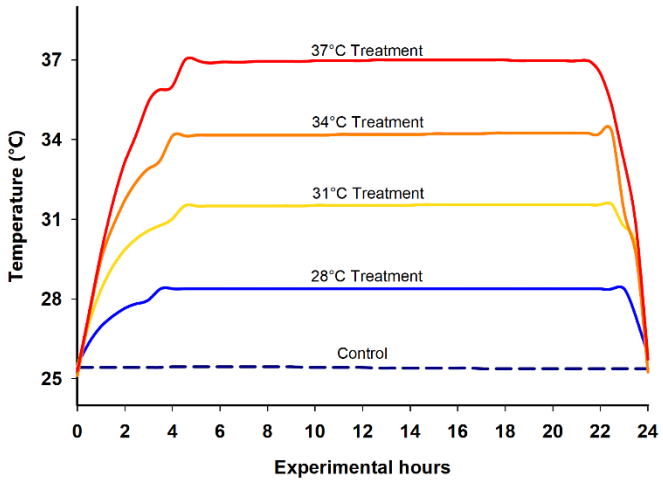

Figure 4. Representative temperature ramping profile for each treatment temperature over 24-hour treatment period.

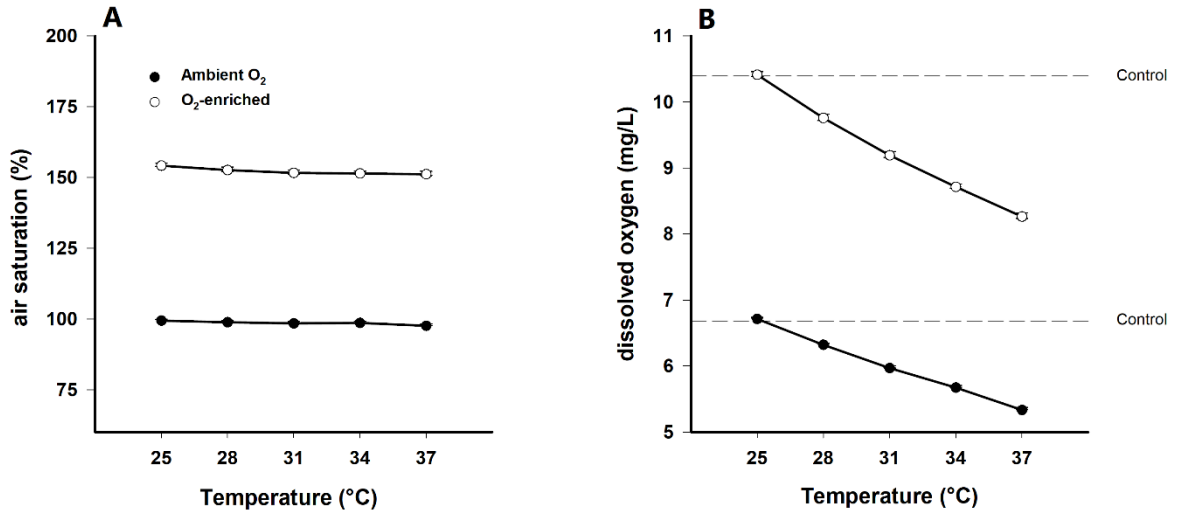

Figure 5. Validation of experimental O_2 manipulation in the water in chambers containing urchins. **A.** Percent air saturation across temperatures for bubbled water in jars maintained under ambient (black circles) and enriched (open circles) O_2. **B.** Concentration of dissolved oxygen across treatment temperatures compared to the control at 25°C. Dashed lines represent DO concentration at 25°C (control) for both oxygen treatments.

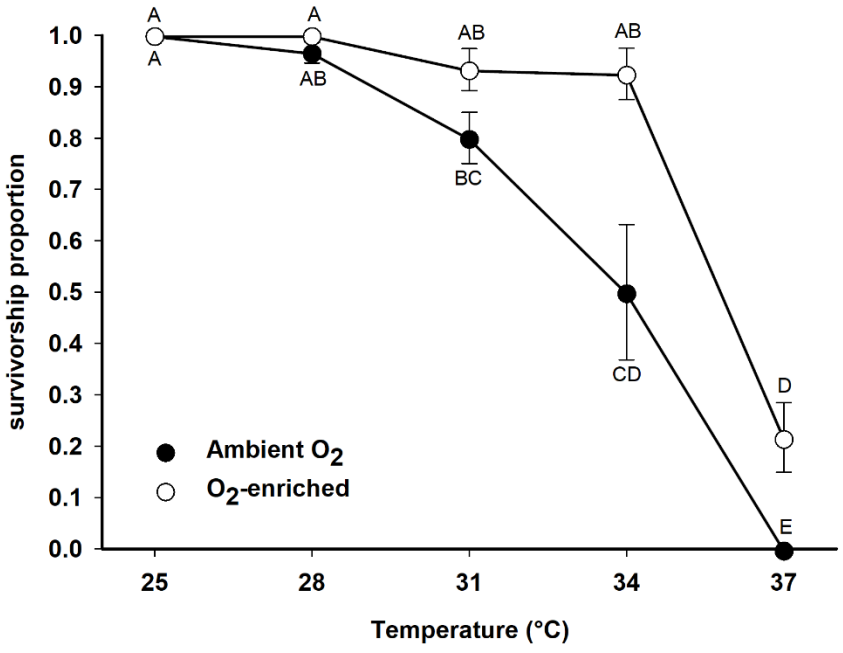

Figure 6. Mean survivorship (±SE) across temperature treatments for each oxygen treatment. Each symbol represents the mean of 5 replicates. Shared letters denote nonsignificant differences (Tukey HSD).

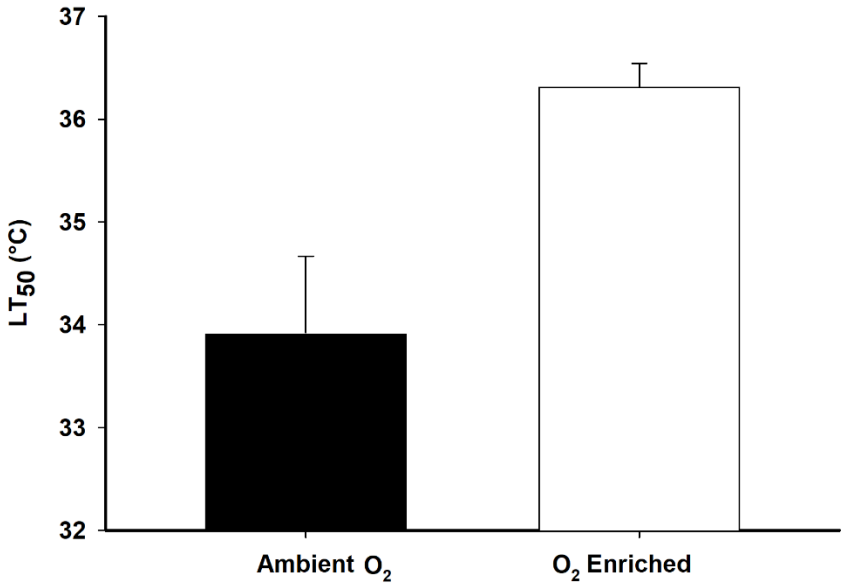

Figure 7. LT_{50} at each oxygen treatment level. Bars represent mean LT_{50} ± standard error (n=5).

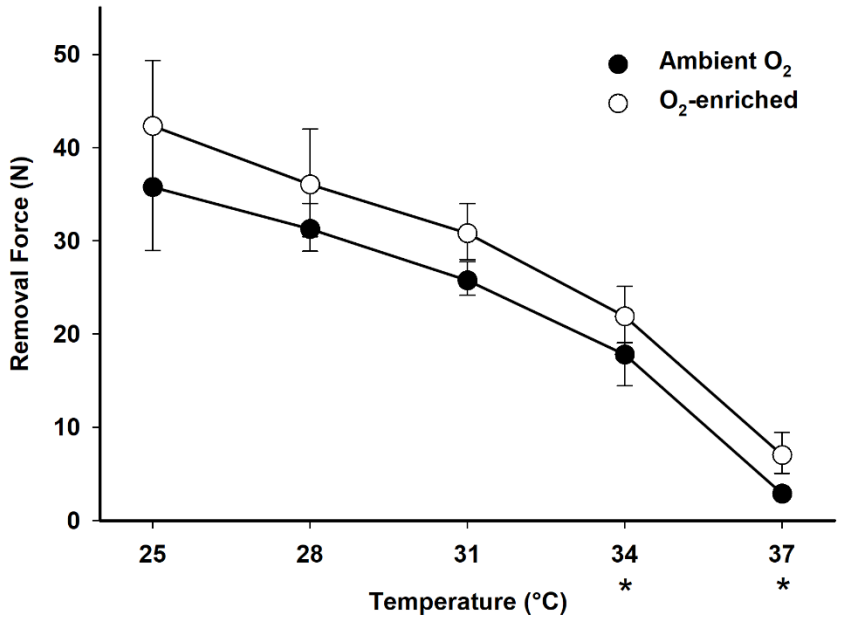

Figure 8. Mean attachment strength (±SE) versus temperature under ambient (filled circles) and enriched (open circles) O_2. Each symbol represents the mean of all urchins under each experimental condition (ambient: 25°C, n=60; 28°C, n=59; 31°C, n=56; 34°C, n=42; 37°C, n=1. enriched: 25-28°C, n=60; 31°C, n=58; 34°C, n=55; 37°C, n=22). Asterisk indicates significant difference of response at treatment temperature from control temperature (P<0.05).

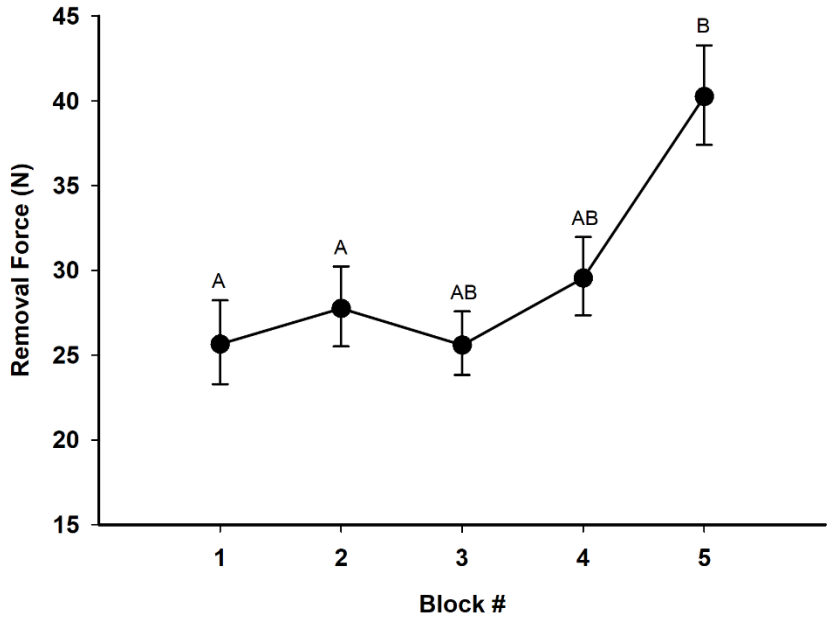

Figure 9. Mean attachment strength (±SE) versus run. Each symbol represents total number of urchins tested in the run (#1, n=98; #2, n=90; #3, n=98; #4, n=92; #5, n=95). Shared letters denote nonsignificant differences between runs (Tukey HSD).

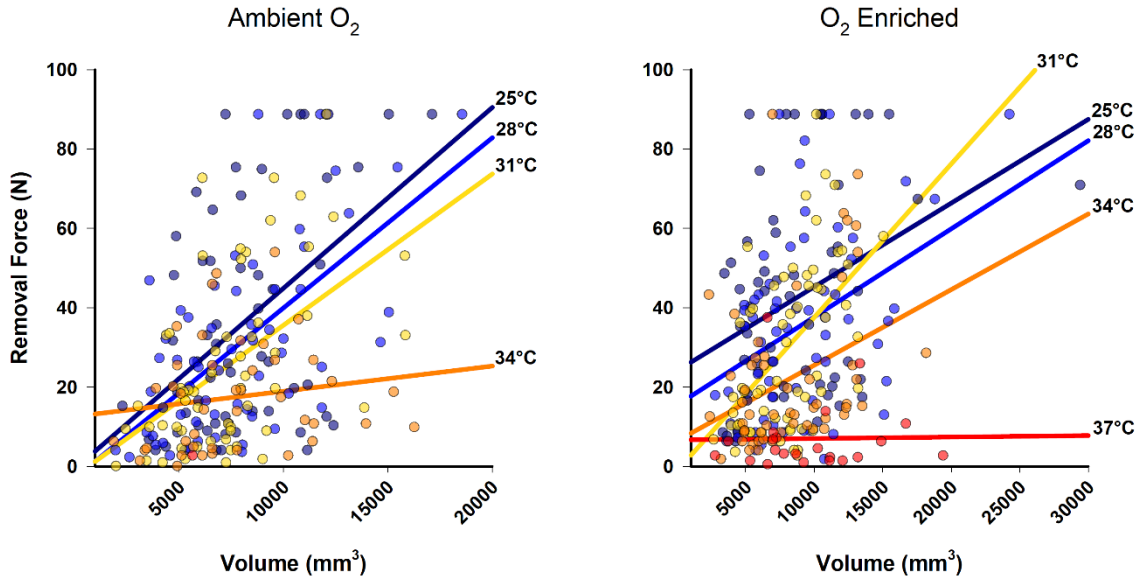

Figure 10. Attachment strength under each oxygen environment versus volume of individual urchins in mm^3 at each treatment temperature; 25°C (purple), 28°C (blue), 31°C (yellow), 34°C (orange), 37°C (red). There is no line of fit in the ambient O_2 treatment at 37°C due to high mortality.

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